J. Parasitol., 90(6), 2004, pp. 1321–1328

A CONSERVED 19-KDA *EIMERIA TENELLA* ANTIGEN IS A PROFILIN-LIKE PROTEIN

R. H. Fetterer, K. B. Miska, M. C. Jenkins, and R. C. Barfield

Animal Parasitic Diseases Laboratory, Animal and Natural Resources Institute, United States Department of Agriculture, Henry A. Wallace Beltsville Agricultural Research Center, Beltsville, Maryland 20705. *e-mail: rfettere@anri.barc.usda.gov*

ABSTRACT: A wide range of recombinant proteins from *Eimeria* species have been reported to offer some degree of protection against infection and disease, but the specific biological function of these proteins is largely unknown. Previous studies have demonstrated a 19-kDa protein of unknown function designated SZ-1 in sporozoites and merozoites of *Eimeria acervulina* that can be used to confer partial protection against coccidiosis. Reverse transcriptase–polymerase chain reaction indicated that the gene for SZ-1 is expressed by all the asexual stages of *Eimeria tenella*. Rabbit antisera to recombinant SZ-1 recognized an approximately 19-kDa protein from extracts of *E. tenella* sporozoites, merozoites, sporulated oocysts, and oocysts in various stages of sporulation. Immunofluorescence antibody staining indicated specific staining of *E. tenella* sporozoites and merozoites. Staining was most intense in the cytoplasm of the posterior end of the parasite. The primary amino acid sequence of the gene for *E. tenella* SZ-1 deduced from the *E. tenella* genome indicated a conserved domain for the actin-regulatory protein profilin. A conserved binding site for poly-L-proline (PLP), characteristic of profilin was also observed. SZ-1 was separated from soluble extract of *E. tenella* proteins by affinity chromatography using a PLP ligand, confirming the ability of SZ-1 to bind PLP. SZ-1 also partially inhibited the polymerization of actin. The current results are consistent with the classification of SZ-1 as a profilin-related protein.

Avian coccidiosis is recognized as a disease with a major economic impact on poultry production causing worldwide losses estimated to be more than 800 million dollars annually (Williams, 1998). Conventional control of poultry coccidiosis has been medicated feed containing polyether–ionophore coccidiostats and other chemical controls (Allen and Fetterer, 2002). Live vaccines consisting of attenuated parasites have also provided a measure of control for avian coccidiosis (Vermeulen et al., 2003). During the past several years, a major goal of basic research on *Eimeria* species has been identifying proteins for developing a subunit recombinant vaccine to control avian coccidiosis. A number of studies have identified proteins or DNA sequences coding for proteins that can convey at least partial protection to coccidia challenges (Jenkins, 1998). Some of the reported antigens have been associated with specific organelles or proteins such as microneme proteins, refractile bodies, and rhoptries (Tomley et al., 1996; Tomley, 1994; Vermeulen, 1998). However, in general, little characterization of the biological or biochemical function of putative antigens has been conducted. Understanding the function of the class of antigens within the parasite may help develop a rational basis for selecting proteins for use in vaccines.

In a previous study (Jenkins et al., 1988; accession number AAA 62795), a clone (SZ-1 complementary DNA[cDNA]) was identified from expression libraries of *Eimeria tenella* sporozoites that encoded a fusion protein expressing part of a 160/240–kDa protein. The purified fusion protein stimulated activated T cells in vitro. Subsequent studies established that the original molecular weight of SZ-1, based on iodination studies, was an overestimate and demonstrated that SZ-1 cDNA coded for a 19-kDa protein that was suggested to be highly conserved in many stages and species of *Eimeria* (Laurent et al., 1994; accession number CAA81335). More recently, an *Eimeria acervulina* expression library screened with an antisera against an 18- to 27-kDa *E. acervulina* protein fraction identified a cDNA clone (3-1E) that was identical with SZ-1 (Lillehoj et al., 2000; accession number AF113613) and represented the same protein. Antibodies against a synthetic peptide deduced from the sequence of 3-1E reacted with a 20-kDa protein in extracts of *E. acervulina* sporozoites and *E. tenella* sporozoites and merozoites. This study also demonstrated that 3-1E/SZ-1 is an immunologically relevant antigen with a potential role in a vaccine development because it stimulated cell-mediated immunity and provided protection to a challenge infection.

In this study, it was found that SZ-1 is present and synthesized in all asexual stages of *E. tenella*. The SZ-1 gene in *E. tenella* was identified and characterized from the genome database and a putative conserved domain in SZ-1, for profilin, an actin regulatory protein, was observed in the amino acid sequence deduced from the gene structure. Based on the biochemical properties of SZ-1 including binding to a poly-L-proline column and inhibition of actin binding, the function of SZ-1 is consistent with the family of profilin molecules.

MATERIALS AND METHODS

Host and parasites

Chickens (80–100 sex-sals, Moyers Hatcheries Inc, Quakertown, Pennsylvania), 4–5 wk of age were infected with 1.0–1.25 $\times$ 10^5 *E. tenella* (Wampler strain) oocysts per bird, placed in feed. On day 7 postinoculation (PI), the birds were killed by cervical dislocation and the ceca were removed. Oocysts were recovered from infected ceca and sporulated as previously described (Fetterer and Barfield, 2003).

For studies of sporulation time course, oocysts were suspended in phosphate-buffered saline (PBS) containing an antibiotic–antimycotic mixture (GIBCO, Gaithersburg, Maryland) and incubated under aeration at 41 C. At the desired time interval (ranging from 0 to 72 hr), an aliquot containing about 1 $\times$ 10^8 oocysts was removed from the incubation flask and centrifuged and the pellet containing oocysts was resuspended in 1.0 ml of 40 mM Tris and stored at -70 C.

Sporozoites were prepared from fully sporulated oocysts (less than 30 days after harvest) as previously described (Fetterer and Barfield, 2003), except that sporozoites were purified by passage through a cellulose filter pad (Fuller and McDougald, 2001). Merozoites were collected from ceca at 108 hr PI from birds inoculated with 3 $\times$ 10^5 sporulated oocysts per bird. Merozoites were isolated and purified as described for sporozoites. Isolated sporozoites and merozoites were resuspended in 40 mM Tris and frozen at -70 C.

Identification of the *Eimeria tenella* SZ-1 gene

To identify the SZ-1 homolog in the genome of *E. tenella*, the entire cDNA encoding this gene (GenBank AF113613) was used to search the *E. tenella* genome using the OmniBlast server located at the Sanger Institute (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/e_tenella/omni). Amino acid alignments were performed using the ClustalX software (Thompson et al. 1997) with minor visual corrections. Conserved

Received 30 December 2003; revised 25 March 2004; accepted 30 March 2004.

domains for profilin were identified using a simple modular architecture research tool (SMART) that allows the identification and annotation of genetically mobile domains and the analysis of domain architectures (Schultz et al., 2000).

Protein extracts

Oocysts (1×10^8) suspended in 1 ml of 40 mM Tris containing a cocktail of proteolytic inhibitors (Complete, Roche Diagnostics, Mannheim, Germany or Proteolytic Cocktail, Sigma, St. Louis, Missouri) were placed in a 1.5-ml capped microfuge tube containing 0.5 g of 0.5 mm glass beads and homogenized with a mini bead-beater (BioSpec Products, Bartlesville, Oklahoma). Soluble extracts were prepared as previously described (Fetterer and Barfield, 2003). Sporozoites and merozoites ($3–5 \times 10^8$) suspended in 0.5 ml of ice cold 40 mM Tris containing a proteolytic cocktail (Sigma) were homogenized by 5, 10-sec bursts with an ultrasonic cell disruptor. Homogenates were centrifuged at 104,000 *g* at 4 C, and supernantants were retained as the soluble extract. In preparations used for 2D-electrophoresis, endonucleases (ribonuclease A, 7 μg/ml, deoxyribonuclease I, 20 μg/ml; Sigma) were added before centrifugation and samples incubated at room temperature for 20 min.

Concentrations of soluble proteins were measured with bicinchoninic acid assay (Pierce, Rockford, Illinois). Bovine serum albumin served as the standard.

Isolation of recombinant SZ-1 and preparation of anti–SZ-1 antisera

The SZ-1 cDNA (Jenkins et al. 1988, GenBank AAA 62795) was excised from pUC19-EASZ240 by *Eco*RI digestion, isolated by agarose electrophoresis, and cloned into *Eco*RI-digested pBAD-HisB expression vector (Invitrogen, Carlsbad, California) using T4 DNA ligase (New England Biolabs Inc., Beverly, Massachusetts). The ligated pBAD-HisB-EASZ240 plasmid was used to transform DH5α *Escherichia coli* and plasmid DNA validated by automated sequencing. Recombinant polyHis-EASZ240 (SZ-1) protein was expressed by a 4-hr treatment with 0.2% arabinose induction of pBAD-HisB-EASZ240–transformed *E. coli* LMG194 cells (Invitrogen). Recombinant protein was extracted under denaturing conditions (3M urea) and was purified by NiNTA affinity chromatography according to the manufacturer's instructions (Invitrogen).

Female NZW rabbits, ≈2 kg and specific pathogen-free, from Covance Research Products (Denver, Pennsylvania) were immunized using the ImmuMax SR adjuvant system (Zonagen, Inc. The Woodlands, Texas; Fetterer and Barfield, 2003) and 200 μg of recombinant SZ-1 given as 2 injections at 30-day intervals. Before immunization, rabbits were bled through the central auricular artery to obtain baseline control sera. At 37 days after immunization, a second blood sample was obtained to determine positive antibody response and titer.

Electrophoresis

Protein samples were analyzed by 1-dimensional (1D) polyacrylamide gel electrophoresis using 1-mm-thick gradient or nongradient mini gels (8×9 cm, 4–12% Bis Tris; Invitrogen) fixed and stained as previously described (Fetterer and Barfield, 2003). Gels were calibrated for molecular weight by comparison with molecular weight standards digitally photographed and analyzed with gel imaging software (Labworks, UVP, Upland, California). Western blotting was performed as previously described (Fetterer and Barfield, 2003).

For 2D-electrophoresis, soluble oocyst proteins were prepared, and separations were performed as previously described (Fetterer and Barfield, 2003). Gels were fixed in acidified methanol, stained with SyproRuby gel stain (Biorad, Hercules, California), and fluorescence of spots observed with a digital camera (UVP). In other experiments, proteins were transferred to polyvinylidene difluoride membranes and a Western blot performed as described for 1D gels. Standards (Pierce, 2D standards) were run in an identical manner to samples and were used to calibrate gels. Gels were analyzed using automated spot detection software (PDQuest, Biorad).

Partial purification of SZ-1

Soluble protein from sporulated oocysts (0.5–2 mg) in 40 mM Tris, pH 8.8, was injected onto a high-performance liquid chromatography anion exchange column (DEAE 5PW, Protein-Pak, 7.5×75 mm steel, Waters, Milford, Massachusetts). The mobile phase consisted of 0.05 M Tris, pH 8.0 (A) and 0.05 M Tris, 1.0 M NaCl, pH 8.0 (B). The column was eluted with solvent A for 10 min followed by a linear gradient from 100% A, 0% B to 50%A, 50%B for 35 min. The flow rate was 1.0 ml per min at 22 C. Aliquots were collected at 1-min intervals (1 ml), and absorbance was monitored with a photodiode array (Waters Model 996) detector. The 1-ml aliquots were pooled into 5 fractions based on the chromatogram. After dialysis against water, fractions were concentrated and 2 μg of protein from each fraction was analyzed by Western blotting with the anti–SZ-1 antibody as described above.

Reverse transcriptase–polymerase chain reaction analysis

All tissues used in RNA isolation were snap frozen after collection and were stored at −70 C until use. Total RNA was isolated from merozoites, sporozoites, sporulated oocysts, and unsporulated oocysts of *E. tenella* using TRIzol (Invitrogen). Each sample was combined with 3 g of diethylpyrocarbonate-treated Pyrex beads (3 mm diameter) (Corning, New York) and 10 ml of TRIzol, then vortexed for 1 min followed by incubation on ice for 1 min. This process was repeated 4 times to ensure complete disruption of the parasite. The remainder of the RNA isolation was carried out using the manufacturer's recommended instructions. Precipitated RNA was resuspended in DNase-RNase–free water (Invitrogen) and stored at −70 C. Before cDNA synthesis, a portion of the *E. tenella* total RNA was treated with Rnase-free DNase I (Invitrogen) using the manufacturer's instructions. cDNA was synthesized from 0.8 μg of total RNA using the recommended instructions provided with the Advantage RT for PCR kit (BD Biosciences Clontech, Palo Alto, California) using oligo dT or random hexamer primers. Profilin transcripts were amplified from 5.0 μl of cDNA with TAP DNA polymerase (CLP, San Diego, California) in the presence of 10 μM primer. Forward primer KM75 (5′ AAATCGCCACGTCGTAGTTC 3′) was used in combination with reverse primer KM76 (5′ GGTCGTTGAAGGTGAAGTCC 3′) to amplify the transcript from *E. tenella* cDNA. The amplifications were carried out as follows. Initial heat activation of polymerase, 95 C for 7 min; denaturation, 94 C for 30 sec; annealing, *E. tenella* 52 C for 30 sec; extension, 72 C for 1 min; and a final extension of 5 min at 72 C. Cycles 2–4 were repeated 30 times.

Immunolocalization

Eimeria tenella sporozoites (SZ) and merozoites (MZ) were washed in (1×) PBS, centrifuged to concentrate, air-dried onto slides, and fixed with 100% methanol (5 min/4 C). Prepared slides were stored at 4 C in airtight containers for up to 2 wk before staining. Specimens were blocked with SuperBlock (Pierce #37515) in PBS–Tween 20 (0.05%), for 30 min at 37 C in a humid chamber and washed 5 min before exposure to primary antibody. Slides were incubated with rabbit anti–SZ-1 and diluted 1:50 in blocking buffer for 1 hr at room temperature inside a humidified chamber. Preimmunization sera at 1:50 were used as negative controls. Secondary antibody, fluorescein-conjugated goat anti-rabbit (Pierce #31572) at 1:100 dilution, was applied for 1 hr. Each incubation was followed by 3×5–min washes. A wet mount was used to apply coverslips to specimens consisting of a 2.5% solution of 1,4 diazobicyclo [2,2,2] octane (Aldrich #D2,780-2) in glycerol (Eschenbacher et al., 1996). Slides were observed under a Zeiss fluorescent microscope using ×400 magnification and a Zeiss #487709 fluorescent filter and photographed with a Nikon DXM-1200 digital color camera.

PLP affinity chromatography

A PLP affinity column was prepared with slight modification of previously described procedures (Edamatsu et al., 1990). Cyanogen bromide–activated sepharose (Sigma) was washed in cold 1-mM HCl for 1 hr. The PLP (100 mg, Sigma) was dissolved in a maximum of 10-ml coupling buffer (CB; 100 mM $NaHCO_3$, 500 mM NaCl) dissolved with very gentle agitation in the presence of the affinity matrix for 2 hr at room temperature with gentle rotation. The unbound ligand was removed by washing with CB, and the unreacted groups were blocked by incubation with 0.2 M glycine for 2 hr at room temp. The coupled matrix was washed with 4 cycles each of CB followed by 0.1 M $NaCH_3O_2$ pH 4 and 0.5 M NaCl. The coupled matrix was stored in 1

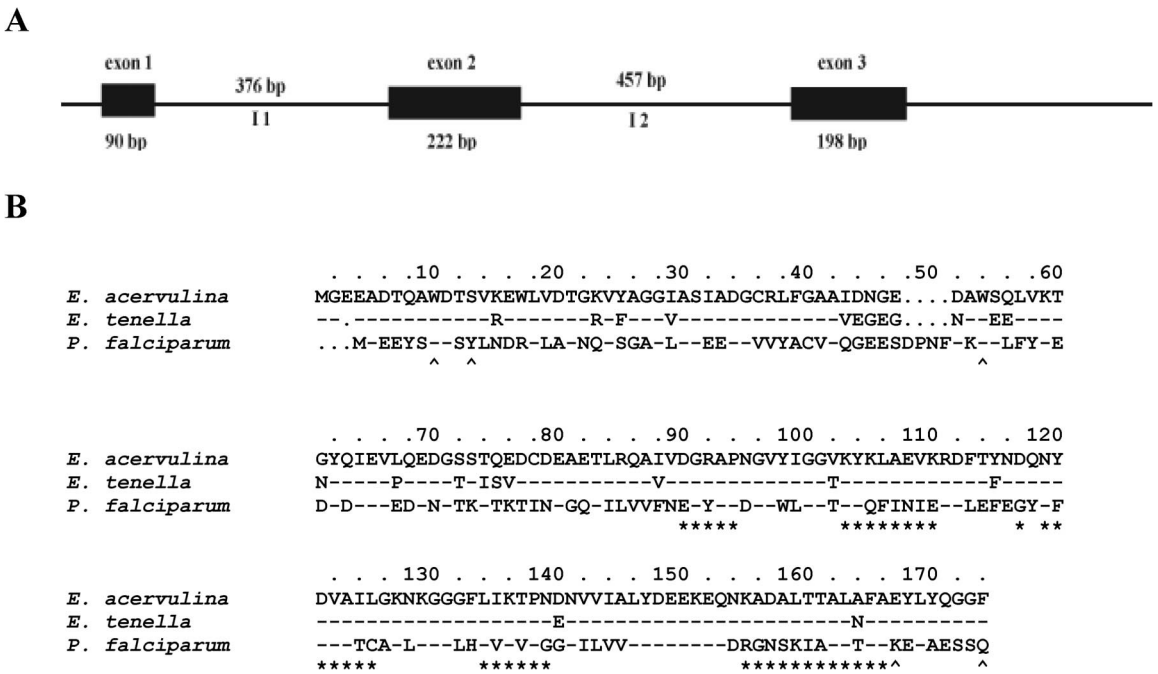

FIGURE 1. Molecular analysis of the *Eimeria tenella* SZ-1 gene. **A.** The structure of gene deduced from the *E. tenella* genomic database. The putative *E. tenella* SZ-1 consists of 170 amino acids, and its predicted molecular weight is 18.5 kDa with an isoelectric point (pI) of 4.1. **B.** Conserved proline and actin-binding sites in SZ-1 from *E. tenella, Eimeria acervulina,* and *Plasmodium falciparum*. Residues important in poly-L-proline binding are indicated by ^. Residues found to be important in actin binding in human profilin II are indicated by *. Sequence identities are indicated by dashes and gaps by dots.

M NaCl. The column was equilibrated in wash buffer (WB; 100 mM Tris, pH 8.0, 150 mM KCl, 1 mM phenylmethane sulfonyl fluoride, and 0.2 mM dithiothreitol [DTT]). About 10 ml of a soluble extract of sporulated oocysts (1 mg/ml) in WB was applied to the PLP affinity column equilibrated with WB. The flow through from the column and was reapplied 5 or 6 times. The column was washed with 100 ml WB, and bound proteins were eluted with WB containing 8 M urea. The absorbance at 280 nm of 2-ml aliquots of eluted fractions was measured and positive fractions pooled concentrated and dialyzed versus WB. Eluted proteins were concentrated and dialyzed versus WB and analyzed by gel electrophoresis followed by staining with SyproRuby or a Western blot probed with the anti–SZ-1 antibody. In some experiments, samples of proteins were precipitated by addition of acetone (90% v/v). Samples were stored overnight at 4 C, centrifuged at 10,000 *g*, and dried under N_2. Samples were dissolved in a sodium dodecyl sulfate (SDS) sample buffer by heating at 70 C before electrophoresis.

Actin spin-down assay

A modified spin-down assay (Edamatsu et al., 1990) was used to assess the ability of SZ-1 to inhibit actin polymerization. Rabbit muscle actin (10 mg/ml, Sigma) was diluted to 0.4 mg/ml in a general actin buffer (5 mM Tris pH 8.0, 0.2 mM $CaCl_2$, 0.5 mM DTT, 0.2 mM adenosine triphosphate [ATP]), incubated on ice for 1 hr and centrifuged at 100,000 *g* at 4 C in a swinging-bucket rotor. The supernatant was collected as the source of the actin monomer. The polymerization was initiated in 250 μl final volume by addition of 25 μl of 10× polymerization buffer (500 mM KCl, 20 mM $MgCl_2$, 10 mM ATP) in the presence or absence of SZ-1. Tubes were incubated at room temperature for 1 hr and then centrifuged at 100,000 *g* at room temperature for 1 hr. A sample of pellet and supernatant from each tube were analyzed by gel electrophoresis and observed with Sypro ruby gel stain (Biorad).

RESULTS

Genomic organization of the *Eimeria tenella* SZ-1 locus

The intron–exon organization of the *E. tenella* SZ-1 gene was determined using the full-length *E. acervulina* cDNA to search the partially completed sequence of the *E. tenella* genome. A single match was found in segment 2257242.c002701728. Contig 1 spanning nucleotides 438–1,780. The deduced sequence of the *E. tenella* SZ-1 gene contains 1,343 nucleotides (Fig. 1A). Because only a single significant match was found during the genome search, it is likely that this gene is encoded by a single locus. The putative *E. tenella* SZ-1 protein consists of 170 amino acids, and its predicted molecular weight and isoelectric point (pI) is 18.5 kDa and 4.1, respectively.

The deduced amino acid sequence of *E. tenella* SZ-1 was aligned with the *E. acervulina* homolog and sequences from a putative homolog of *Plasmoduim falciparum* (Fig. 1B). The amino acid identity between *E. acervulina* and *E. tenella* SZ-1 is 86%. Human profilin II contains 5 PLP-binding sites (Nodelman et al., 1999). In *Eimeria* sp., 4 of these sites are either identical or conserved with the human sequence (Fig. 1B). The actin-binding sites of human profilin II are shown in Figure 1B. These sites in other species, including *Eimeria* spp., show very little conservation.

Expression of SZ-1

On Western blots of recombinant ^SZ-1 expressed in *E. coli*, rabbit anti-sera prepared against recombinant SZ-1 recognized a predominant 28-kDa protein corresponding to the recombinant SZ-1 as well as other proteins of both higher and lower molecular weight. When soluble proteins from *E. tenella* oocysts (after 0–72 hr of sporulation), sporozoites, and merozoites were screened with the anti–SZ-1, a single 19-kDa band was observed in all the stages (Fig. 2A). A Western blot of a 2D analysis of soluble proteins from *E. tenella* sporulated oocysts

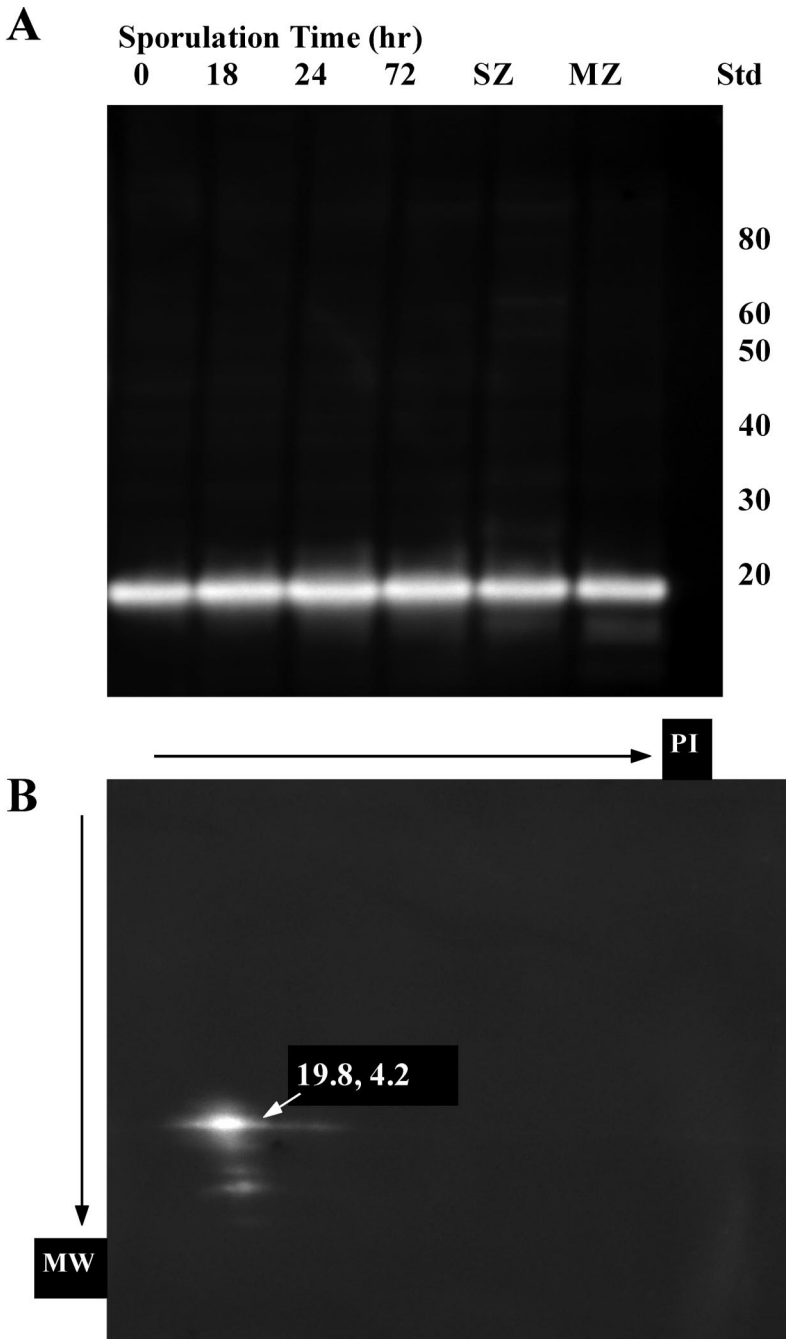

FIGURE 2. Expression of SZ-1 in the developmental stages of *Eimeria tenella*. **A.** Expression of SZ-1 in the developmental stages of *E. tenella* by Western blot (WB). Proteins (5 μg per lane) were separated on 4–12% Bis–Tris mini gels and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were stained with 1:500 dilution of rabbit anti–SZ-1 and 1/100,000 dilution goat anti-rabbit horseradish peroxidase (HRP) secondary antibody and observed after treatment with luminol reagent. **B.** The 2-D gel electrophoresis of soluble proteins from *E. tenella* sporulated oocysts. Proteins (50 μg/strip) were separated in first dimension on IPG strips with a nonlinear pH gradient from 3–10. The second dimension was resolved with an SDS gradient gel transferred to the PVDF membrane and probed with an anti–SZ-1 antibody as described above.

indicated a predominant spot with a molecular weight of 19.4 kDa and pI of 4.2 recognized by anti–SZ-1 antisera (Fig. 2B).

SZ-1 transcripts were amplified from *E. tenella* cDNA synthesized from sporozoites, merozoites, and oocysts at different sporulation time points. The primer pairs used to amplify SZ-1 from *E. tenella* were designed to span an intron to differentiate cDNA-amplified products from genomic DNA (Fig. 3). The expected size of products amplified from cDNA was 149 bp, whereas those amplified from genomic DNA were 606 bp in length (Fig. 3). The reverse transcriptase–polymerase chain reaction results shown in Figure 3 indicate that the SZ-1 gene is expressed in all developmental stages tested.

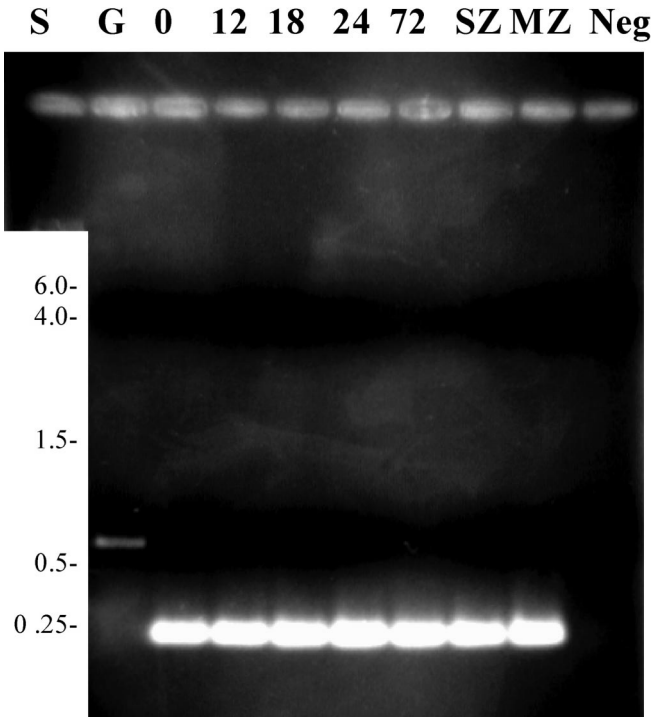

FIGURE 3. The expression of the SZ-1 gene in the developmental stages of *Eimeria tenella*. SZ-1 transcripts were amplified from *E. tenella* cDNA synthesized from oocysts at different sporulation time points (0–72 hr) as well as from sporozoites (SZ) and merozoites (MZ). The expected size of products amplified from cDNA was 149 bp, whereas those amplified from genomic DNA (lane G) were 606 bp in length. N = negative control (no cDNA).

Immunolocalization

When sporozoites were stained with fluorescein-conjugated anti–SZ-1 antibody, intense but often irregular staining was noted in the posterior of the parasite (Fig. 4A). An intense stain was also noted on the extreme anterior of the sporozoite. With respect to the merozoites, the posterior regions stained very intensely and in a uniform manner (Fig. 4B). Little staining of the merozoite anterior end was noted. Neither the sporozoites nor the merozoites stained with prebleed antisera (data not shown).

PLP affinity chromatography

The specifically bound proteins that were eluted from a PLP affinity column with 8 M urea consisted primarily of a dominant band with a molecular weight of about 19 kDa with a second less prominent band migrating at 150 kDa (lane 2, Fig. 5A). Western blots with anti–SZ-1, however demonstrate intense reactivity with the 19-kDa band only (lane 2, Fig. 5B). A 19-kDa protein of much lesser intensity was also observed in the unbound protein fraction (lane 1, Fig. 5B).

Actin spin-down assay

An actin spin-down assay was conducted to determine if SZ-1 could inhibit actin polymerization in vivo (Fig. 6). Both with and without addition of SZ-1, actin could be detected on SDS gels in both the unpolymerized (supernatant) and polymerized (pellet) fractions. However, addition of SZ-1 inhibited the degree of polymerization. In the 2 controls, 78 and 76% of the actin was polymerized and 22 and 24% remained unpolymerized. The addition of SZ-1 resulted in 37 and 39% of actin polymerized, whereas 63 and 61% of actin was unpolymerized.

DISCUSSION

The present results demonstrate that the gene designated SZ-1 originally described from *E. acervulina* (Jenkins et al., 1988) is also present in *E. tenella* and codes for an approximately 19-kDa soluble protein that is highly conserved in all developmental stages of *E. tenella* examined. Supporting this conclusion are observations that anti–SZ-1 antisera made against recombinant SZ-1 from *E. acervulina* recognizes a 19-kDa protein in the asexual stages of *E. tenella* and that the SZ-1 gene

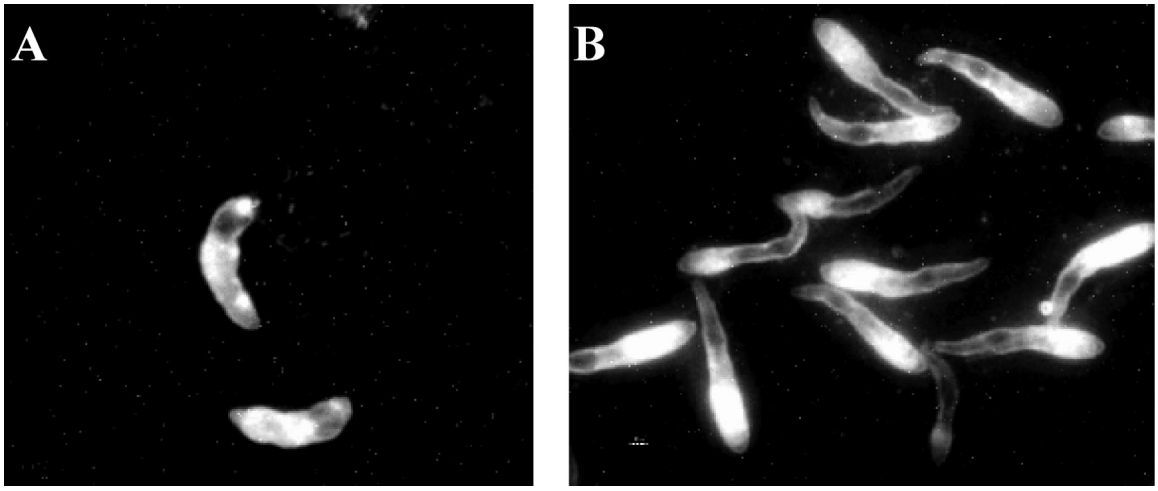

FIGURE 4. Immunofluorescence staining of whole mounts of *Eimeria tenella* sporozoites (**A**) or merozoites (**B**). Whole mounts of parasites were stained with 1:100 dilution of rabbit anti–SZ-1 antisera followed by goat anti-rabbit antibody conjugated to fluorescein (×400). Arrows indicates locations of staining. A sample from the same preparation stained with control (nonimmunized) rabbit sera had little or no reactivity with parasites (not shown).

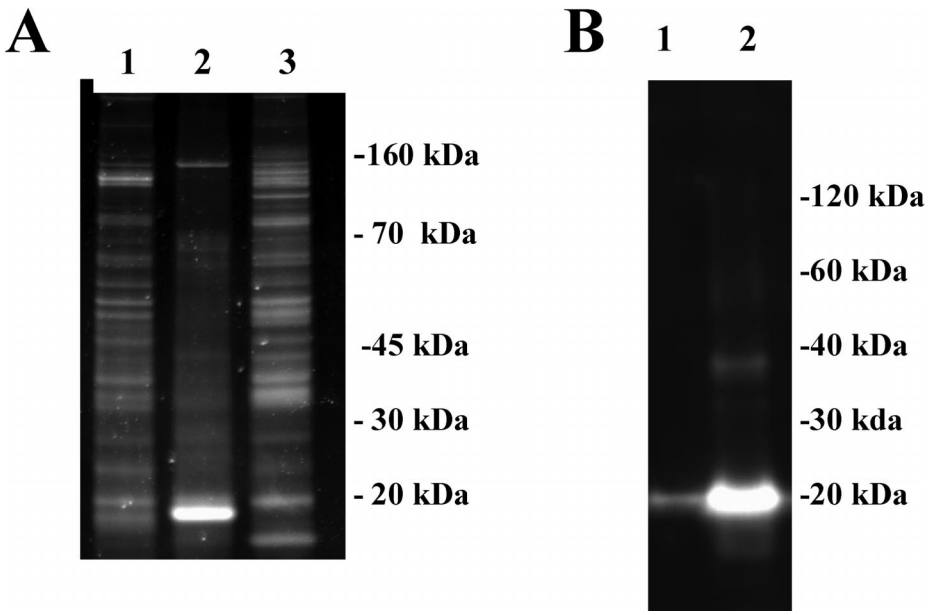

FIGURE 5. Poly-L-proline (PLP) affinity chromatography of soluble extracts of *Eimeria tenella* sporulated oocysts. **A.** Protein (5 μg lane) either unbound to the column (lane 1), eluted with 8 M urea (lane 2), or unfractionated (lane 3) were separated electrophoretically. Gels were stained with SyproRuby and observed with UV transillumination. **B.** Western blot of proteins (0.5 μg per lane) eluted with 8 M urea (lane 1) or unbound to PLP affinity column screened with 1:500 dilution of rabbit anti–SZ-1.

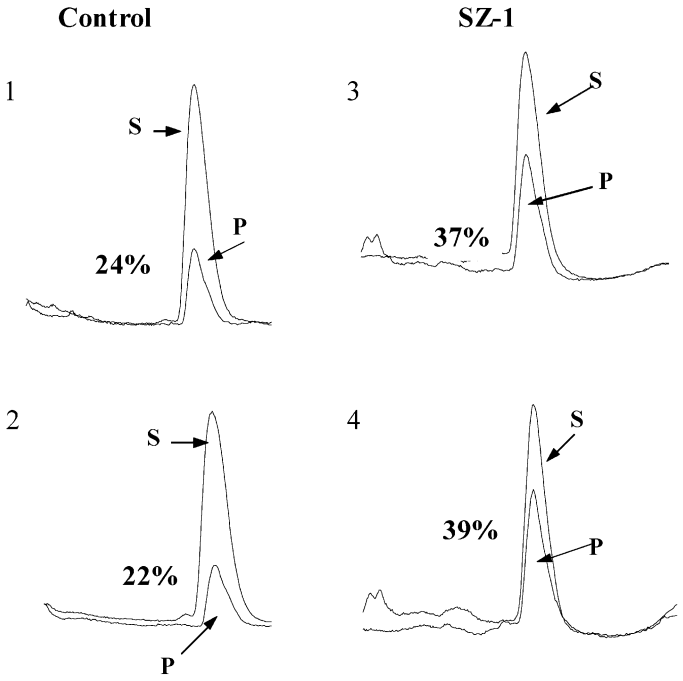

FIGURE 6. A spin-down assay to detect inhibitions of actin polymerization by SZ-1. Actin monomer was polymerized in the absence (1 and 2) or presence (3 and 4) of partially purified SZ-1. Absorbance scan of electrophoretic separation of samples after polymerization and centrifugation to separate polymerized (pellet, P) and unpolymerized (supernatant, S) actin. In the control, 24 and 22% of the actin was unpolymerized, whereas in the presence of SZ-1 the amount in unpolymerized fraction was increased to 39 and 37%.

is expressed during *E. tenella* development. In addition, the pI and molecular weight of SZ-1 measured by electrophoresis are in reasonable agreement with those based on amino acid sequence predicted from the genomic sequence. Sequence identity observed between *Eimeria* spp. and *Plasmodium* spp. are consistent with the previous observation that the SZ-1 gene is conserved within *Eimeria* spp. as well as some other apicomlexans (Laurent et al., 1994).

The function of SZ-1 within *Eimeria* spp. was previously unknown. However, the ubiquitous expression of SZ-1 within all developmental stages examined suggests the function basic to parasite maintenance. The soluble nature of the protein further suggests that the native protein is cytoplasmic in location. Analysis of the predicted amino acid sequence indicates that SZ-1 has a conserved domain characteristic of the actin regulatory protein, profilin. Profilins are soluble cytoplasmic proteins that are 11–16 kDa in size and are widely distributed within animals and plants, where they play a key regulatory role in actin polymerization (Schluter et al., 1997). Although sequence homology varies widely, a key feature of profilins is a conserved PLP-binding site. Binding sites for actin, polyphosphoinositides, phospotidylinositol 4,5-bisphosphate, and possibly other ligands are also present (Schluter et al., 1997). Amino acid sequence alignment of SZ-1 from *Eimeria* spp. with known profilins indicates conservation of PLP-binding sites, which are often used for the purification of profilins from a wide variety of biological sources (Schluter et al., 1997). In this study, we demonstrated the ability of a 19-kDa protein from soluble extracts of *E. tenella* sporulated oocysts that reacts strongly with anti–SZ-1 to bind PLP through affinity chromatography, consistent with that previously observed for profilins (Schluter et al., 1997).

Besides binding proline, profilins also bind actin and presumably regulate actin polymerization by this interaction (Schluter et al., 1997). In this study, addition of partially purified SZ-1 to a solution of actin monomers effectively reduced the polymerization of actin. These results support the conclusion that SZ-1 has some ability to bind actin and, therefore, it acts as an actin-regulator protein. However, the inhibition of actin polymerization was only partial and did not seem to increase when higher concentrations of SZ-1 were used in the polymerization assay (data not shown). Other assays of actin polymerization–binding may be needed to determine the interaction of SZ-1 with actin. Also, mammalian muscle actin was used in the assay described here. Actin from nonmuscle sources may be a more effective substrate for *E. tenella* profilins (Edamatsu et al., 1990).

Profilin is expected to be concentrated in the cytoplasm within areas of highly dynamic actin filaments (Schluter et al., 1997). In this study, primarily the posterior cytoplasm of both SZ and MZ was stained with anti–SZ-1; however, the anterior tip of SZ was also stained, suggesting some interaction with the apical complex and proteins associated with cell invasion. Our observations are consistent with another report that concluded a cytoplasmic location for SZ-1 in sporozoites from 3 *Eimeria* species (Laurent et al., 1994), although others have indicated a surface location for SZ-1 (Jenkins et al., 1988; Lillehoj et al., 2000). The differences in these reports could be caused by varying degrees of permeablization of parasites affected by fixation techniques, although localization of SZ-1 near or on the parasites surface cannot be eliminated.

Profilins have been widely described from many groups of organisms, but a homolog of profilin has not been previously reported in apicomplexans (Morrisette and Sibley, 2002). Actin-associated proteins isolated by F-actin affinity chromatography have been described in *Plasmodium* spp., although their function is not known (Tardieux et al., 1998). More relevant to the current results, a novel G-actin–binding protein, designated toxofilin, has been characterized in *Toxoplasma gondii* tachyzoites (Poupel et al., 2000). Toxofilin, a 28-kDa basic protein, was isolated by actin affinity and was shown to play a role in the regulation of actin polymerization, but it lacks any sequence or domain homology with profilins or SZ-1 However, a sequence with a high degree of homology to SZ-1 has been reported from *T. gondii* expressed sequence tags (Wan et al., 1996).

The relationship between the profilin function of SZ-1 and its potential role as a protective antigen is unclear. It may be that SZ-1 is simply an abundant soluble protein found in all developmental stages resulting in uncontrolled interaction with the host immune system. It is unlikely that profilins would be secreted spontaneously. Therefore, the release of SZ-1 on destruction of parasites within the host is the likely route of contact of SZ-1 with the host immune system. Profilins from plants are potent allergens causing about 20% of food allergies in some patients (Valenta et al., 1991). It is possible that SZ-1 may have immunostimulatory properties that assist the parasite to escape host immune responses.

The current results are consistent with a hypothesis that the *Eimeria* spp. protein, designated SZ-1, is related to the family of profilins previously described in other organisms. The presence of a profilin domain, PLP-binding site within the proteins, and the demonstration of the binding affinity to PLP as well as the partial inhibition of actin polymerization support this hypothesis. Further investigations of the biochemical and biological properties of SZ-1 within *Eimeria* spp. will be needed to identify other actin regulatory proteins.

ACKNOWLEDGMENTS

The authors are grateful for the expert technical assistance of Gary Wilkins, Jennifer Herrmann, and Mark Paape. We are also indebted to M. Shirley for granting permission to use the *E. tenella* genome sequence available at the Sanger Institute website and D. Zarlenga for critical reading of the manuscript. This work was supported by USDA/ARS CRIS project number 1265-31320-070-00D.

LITERATURE CITED

ALLEN, P. C., AND R. H. FETTERER. 2002. Recent advances in biology and immunobiology of *Eimeria* species and in diagnosis and control of infection with these coccidia parasites of poultry. Clinical Microbiological Reviews **15:** 58–65.

EDAMATSU, M., N. IRONO, AND Y. WATANBE. 1990. Purification and characterization of *Tetrahyamena* profilin. Biochemical and Biophysical Research Communications **170:** 957–962.

ESCHENBACHER, K.-H., P. EGGLII, M. M. WALLACH, AND R. BRAUN. 1996. Characterization of 14 kDa oocyst wall proteins of *Eimeria tenella* and *E. acervulina*. Parasitology **112:** 169–176.

FETTERER, R. H., AND R. C. BARFIELD. 2003. Characterization of a developmentally regulated oocyst protein from *Eimeria tenella*. Journal of Parasitology **89:** 553–564.

FULLER, A. L., AND L. R. MCDOUGALD. 2001. Cell membrane labeling of *Eimeria tenella* sporozoites with the fluorescent dye PKH-67 GL for tracking parasite-host interactions. Parsitology Research **87:** 521–525.

JENKINS, M. C. 1998. Progress on developing a recombinant coccidiosis vaccine. International Journal for Parasitology **28:** 1111–1119.

———, H. S. LILLLEHOJ, AND J. B. DAME. 1988. *Eimeria acervulina*: DNA cloning and characterization of recombinant sporozoite and merozoite antigens. Experimental Parasitology **66:** 96–107.

LAURENT, F. C., C. BOURDIEU, M. KAZANJI, P. YVORE, AND P. PERRY. 1994. The immunodominant *Eimeria acervulina* sporozoite antigen previously described as p160/p240 is a 19 kilodalton antigen present in several *Eimeria* species. Molecular and Biochemical Parasitology **63:** 79–86.

LILLEHOJ, H. S., K. D. CHOI, M. C. JENKINS, V. N. VAKHARIA, KI. D. SONG, J. Y. HAN, AND E. P. LILLEHOJ. 2000. A recombinant *Eimeria* protein inducing interferon-gamma production: Comparison of direct gene expression systems and immunization strategies for vaccination against coccidiosis. Avian Diseases **44:** 379–389.

MORRISETTE, N. S., AND L. D. SIBLEY. 2002. Cytoskeleton of Apicomlexan parasites. Microbiology and Molecular Biology Reviews **66:** 21–38.

NODELMAN, I. M, G. D. BOWMAN, U. GINDER, AND C. E. SCHUTT. 1999. X-ray structure determination of human profilin II: A comparative structural analysis of human profilins. Journal of Molecular Biology **294:** 1271–85.

POUPEL, O., J. BOLETI, S. AXISA, E. COUTURE-TOSI, AND I. TARDIEUX. 2000. Toxofilin, a novel actin binding protein from *Toxoplasma gondii*, sequesters actin monomers and caps actin filaments. Molecular Biology of the Cell **11:** 355–368.

SCHLUTER, K., B. JOCKBUSCH, AND M. ROTHKEGEL. 1997. Profilins as regulators of actin dynamics. Biochemica et Biophysica Acta **1939:** 97–109.

SCHULTZ, J. R., R. COPLEY, T. DOERKS, C. P. PONTING, AND P. BORK. 2000. SMART: A web-based tool for the study of genetically mobile domains. Nucleic Acids Research **28:** 231–234.

TARDIEUX, I., I. BAINES, M. MOSSAKOWSKA, AND G. E. WARD. 1998. Actin-binding proteins of invasive malaria parasites and the regulation of actin polymerization by complex of 32/34-kDa proteins associated with heat shock protein 70 kDa. Molecular and Biochemical Parasitology **93:** 295–308.

THOMPSON, J. D., T. J. GIBSON, F. PLEWNIAK, F. JEANMOUGIN, AND D. G. HIGGINS. 1997. The ClustalX windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research **24:** 4876–82.

TOMLEY, F. M. 1994. Characterization of rhoptry proteins of *Eimeria*

tenella sporozoites: Antigenic diversity of rhoptry epitopes within species of the genus *Eimeria* and among three asexula generations of a single species of *Eimeria tenella*. Infection and Immunity **62:** 4656–4658.

———, J. M. BUMSTEAD, K. J. BILLINGTON, AND P. P. J. DUNN. 1996. Molecular cloning and characterization of a novel microneme protein (Etmic-2) from the Apicomlexan protozoan parasite, *E. tenella*. Molecular Biochemistry and Parasitology **79:** 195–206.

VALENTA, R., M. DUCHENE, K. P. BRETTEBACH, H. ETTENBURGER, C. SILLABER, P. VALENT, P. BETTELHEM, M. BRETENBACH, H. RUMPOLD, D. KRAFT, AND D. O. SCHEINER. 1991. Identification of profilin as a novel pollen allergen; IgE autoreactivity in sensitized individuals. Science **253:** 557–560.

VERMEULEN, A. N. 1998. Progress in recombinant vaccine development against coccidiosis. A review and prospects into next century. International Journal of Parasitology **28:** 1121–1130.

———, C. SCHAAP, AND TH. P. M. SCHETTERS. 2003. Control of coccidiosis in chickens by vaccination. Veterinary Parasitology **100:** 13–20.

WAN, K. L., J. M. BLACKWELL, AND A. J. AJIOKA. 1996. *Toxoplasmo gondii* expressed sequence tags: insight into tachyzoite gene expression. Molecular and Biochemical Parasitology **75:** 179–186.

WILLIAMS, R. B. 1998. Epidemiological aspects of the use of live anticoccidial vaccines for chickens. International Journal for Parasitology **28:** 1089–1098.